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Insect diets as mixtures: Optimization for a polyphagous weevil

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ABSTRACT

Development or improvement of artificial insect diets can be tedious, convoluted and often under-appreciated. Using *n*-dimensional mixture designs, we identified a set of response-optimized meridic diets that contain fewer ingredients than the current commercial diet for *Diaprepes abbreviatus*, a polyphagous weevil pest of the Caribbean and southern U.S. A diet blend optimized to produce maximum adult weight was predicted to produce adult *D. abbreviatus* that weigh 28% more compared with adults reared on the standard commercial diet. Diet blends that produced greater individual adult weights resulted in lower survival compared with those blends that yielded adults of more modest proportions. In contrast, a simplified high cottonseed meal blend produced smaller adults more similar to field-collected individuals, and produced the greatest number of adults and the greatest biomass at relatively low cost compared with diets that yielded adult weevils of greater weight. We think that many insect-rearing programs would benefit from application of mixture design methods to situations where diet optimization is desired for researcher-selected criteria. This approach is broadly applicable to any problem that can be conceptualized as a mixture problem.

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1. Introduction

In the preface to his recent book on insect diets, Allen Cohen (2004) suggested, "...the success of entomology over the past century is founded on our ability to rear insects on artificial diets. Much of future entomology will likely continue to depend on diet-based programs." While Cohen emphasized the need to understand how and why diets work or fail, many practitioners of biological control, host plant resistance, insect ecology, and related disciplines would benefit from simplified methods to optimize already existing diets based on responses determined by the subject matter. For example, applied entomologists studying insect behavior may be interested in producing diet-reared insects that closely resemble field-collected insects. Some rearing operations, e.g., sterile release, will be interested in producing as many adult insects as possible on a given amount of diet in the shortest time possible. Others will be more interested in producing the largest individual insects possible or minimizing expensive ingredients. With an understanding of the principal diet components affecting insect growth and development on diet mixtures, optimal diet blends for individual insect species could be identified to meet researchers' needs. Instead of a single diet for a given species, researchers could specify the desired results and then calculate the nutritional composition of an appropriate diet.

Modern multivariate geometric designs for mixture experiments are well suited for insect diet formulation problems. Most insect diets are complex mixtures of vitamins, salts, preservatives, and nutrients (carbohydrates, lipids and proteins). The traditional approach to determine the effect of varying the doses of multiple components requires large factorial experiments resulting in large numbers of treatment combinations and multiple interaction terms that are difficult to interpret. Furthermore, factorial designs allow parallel variation of variables, making them inappropriate for mixture problems wherein fillers must be used to obtain all of the experimental diets (Ruohonen and Kettunen, 2004).

An insect diet experiment can be conceptualized as an *n*-dimensional space with each diet component representing a single vector. Current computer hardware and software make it possible to design, execute and analyze highly efficient experiments that systematically sample the *n*-dimensional experimental design space, identify key drivers for specific response variables, and generate mathematical equations that describe multiple response variables. The ideal result is a set of equations that describe insect responses to varying blends of diet components, thereby allowing researchers to choose the most desirable outcome as defined by the objectives of the research or application.

Our immediate interest was to answer a set of questions related to the rearing of *D. abbreviatus*, a major polyphagous pest of citrus,

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ornamentals and other crops in the Caribbean and the southern U.S. (Lapointe et al., 2007): could the diet be simplified and made more economical; could diets be identified that would satisfy different individual criteria or combinations of criteria; could the diet be designed to produce adult weights similar to field-collected insects; and could we mathematically describe the multidimensional space encompassing all combinations of principal diet ingredients, thereby relating the organism to its diet components in Euclidian space? We illustrate how this approach can produce multiple insect diets optimized for any measured criterion with a minimum of experimentation.

2. Methods

2.1. Insect rearing

All stages of *D. abbreviatus* were reared at the U.S. Horticultural Research Laboratory, Fort Pierce, FL following methods already described (Lapointe and Shapiro, 1999). Eggs were collected from caged adults on wax-paper strips (Wolcott, 1933) and allowed to hatch in plastic containers. Standard procedure for preparing purchased diet (Table 1) for larval development was as follows: 40 l of water were combined with 725 g agar and heated to near boiling. While stirring, 9.5 kg of commercially prepared insect diet [product no. F1675, Bio-Serv, Inc., Frenchtown, NJ, similar to that developed by Beavers (1982)] were added to the water/agar mixture, mixed, and heated to boiling. Methyl paraben (9 g dissolved in 10 ml 95% EtOH) and 9 g of benzoic acid in solution with boiling deionized water were added as preservatives. After 10 min of boiling, ~15 ml of diet was dispensed into 30-ml plastic cups and allowed to cool and dry in a laminar flow hood. Neonate larvae were surface sterilized for ~2 min. in a 0.25% hypochlorite solution, rinsed with deionized water, and placed in cups with diet. Each diet cup was infested with approximately 12 neonate larvae (<24 h old) and 15 cups were infested for each experimental diet (treatment). Diet cups were capped and placed in trays enclosed in zip-lock plastic bags and held in a dark environmental chamber at 25 °C and 60–70% RH. Humidity within the diet cups held in sealed plastic bags probably exceeded 95% (Lapointe, 2000). Cups were opened 4 weeks after infestation and larvae were counted and weighed. A total of 30 larvae per treatment were randomly selected and transferred to fresh diet cups to complete development (one larva per cup). Larvae pupated in the diet cups. Cups were inspected on weekdays to determine date of pupation, pupal duration, and date of adult emergence. The date and time of an observed event (e.g., death, pupation, adult emergence) were calculated as the midpoint between the times of the observed change and the previous inspection. Time to pupation, pupal period, time to adult emergence, and adult weight and sex were recorded for 6 months from date of infestation with neonates. Insects that failed to pupate within 6 months were discarded.

Previous experimentation showed that the F1675 diet could be diluted with cellulose with no significant reduction in insect weight gain or survival (Lapointe et al., 2003). Therefore, the amount of cellulose was increased to 45% in all experimental mixes compared with 31% in the F1675 diet. The undiluted commercial diet, and commercial diet with cellulose increased to 45% of dry ingredients were included as controls in all experimental blocks.

2.2. Experimental design

A two-step approach was utilized to identify the most important diet ingredients (principal drivers) of the F1675 diet and then to optimize the diet in relation to these principal drivers for specific response variables. This involved an initial screening design (Cornell, 2002) that identified the most important ingredients from eight candidate components in the standard diet. We then identified the three primary drivers and constructed a three-component mixture design that explored the interplay between these ingredients and identified optimal mixtures for several responses of interest.

2.3. Screening design

The commercial F1675 diet consists of a total of 35 ingredients (Table 1). The vitamin and salt blends were treated as single components and no effort was made to de-convolute these mixtures. Ascorbic acid, cholesterol, choline chloride, methyl paraben, and sorbic acid were kept constant at the levels in the F1675 diet. The screening design was then formulated to vary eight diet components: cottonseed meal, soy protein isolate, vitamin mix, casein, wheat germ, cornstarch, salt mix, and sucrose. A screening design was created with Design-Expert (v7.0.3, State-Ease, Inc., Minneapolis, MN) as a simplex, linear mixture that consisted of vertex, center, seven-blend, and axial check blend treatment points (Table 2; Anderson and Whitcomb, 2005).

Table 1
Ingredients contained in the commercial Bio-Serv F1675 blend of dry diet components used to rear *D. abbreviatus* and the composition of two component blends, the Vanderzant vitamin mix (Bio-Serv product #9796) and the Wesson modified salt mix (Bio-Serv product #9798)

F1675 diet		Vitamin mix		Salt mix	
Ingredient	g/kg	Ingredient	g/kg	Ingredient	g/kg
Cellulose	312.6	Vit. E acetate (50%)	0.5	Calcium carbonate	3.4
Cottonseed meal	254.6	Ascorbic acid	8.5	Copper sulfate	0.0
Soy protein, isolate	105.9	Biotin	0.0	Ferric phosphate	0.2
Vanderzant mix	31.6	Calcium pantothenate	0.0	Manganese sulfate (anhyd.)	0.0
Casein	71.3	Cholin dihydrogen citrate	3.3	Magnesium sulfate (anhyd.)	1.5
Wheat germ	61.1	Folic acid	0.0	Potassium aluminum sulfate	0.0
Cornstarch	44.8	I-Inositol	0.6	Potassium chloride	2.0
Methyl paraben	15.3	Niacin	0.0	Potassium dihydrogen phosphate	5.1
Ascorbic acid	5.1	Pyridoxine HCl	0.0	Potassium iodide	0.0
Sorbic acid	5.1	Riboflavin	0.0	Sodium chloride	1.7
Cholesterol	3.1	Thiamin HCl	0.0	Sodium fluoride	0.0
Wesson salt mix	16.3	B-12 (0.1%)	0.1	Tricalcium phosphate	2.4
Sucrose	71.3	Sucrose	18.4		
Choline chloride	2.0				
Total	1000.0		31.6		16.3

Table 2
Proportions of eight diet components varied in a seven-dimensional, mixture screening design to rear *D. abbreviatus*

Diet blend #	Casein	Cornstarch	Cottonseed meal	Salt mix	Soy protein	Sucrose	Vitamin mix	Wheat germ
1	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
2	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	0.063	0.063	0.063	0.063	0.063	0.063	0.563	0.063
4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
5	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
6	0.143	0.143	0.143	0.000	0.143	0.143	0.143	0.143
7	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000
8	0.143	0.143	0.000	0.143	0.143	0.143	0.143	0.143
9	0.063	0.063	0.063	0.563	0.063	0.063	0.063	0.063
10	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.000
11	0.000	0.143	0.143	0.143	0.143	0.143	0.143	0.143
12	0.143	0.143	0.143	0.143	0.000	0.143	0.143	0.143
13	0.063	0.063	0.563	0.063	0.063	0.063	0.063	0.063
14	0.143	0.143	0.143	0.143	0.143	0.000	0.143	0.143
15	0.143	0.143	0.143	0.143	0.143	0.143	0.000	0.143
16	0.063	0.563	0.063	0.063	0.063	0.063	0.063	0.063
17	0.563	0.063	0.063	0.063	0.063	0.063	0.063	0.063
18	0.063	0.063	0.063	0.063	0.063	0.063	0.063	0.563
19	0.063	0.063	0.063	0.563	0.063	0.063	0.063	0.063
20	0.143	0.143	0.143	0.000	0.143	0.143	0.143	0.143
21	0.063	0.063	0.063	0.063	0.063	0.063	0.563	0.063
22	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
23	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000
24	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000
25	0.143	0.000	0.143	0.143	0.143	0.143	0.143	0.143
26	0.143	0.143	0.143	0.143	0.143	0.143	0.000	0.143
27	0.063	0.063	0.063	0.063	0.563	0.063	0.063	0.063
28	0.063	0.063	0.063	0.063	0.063	0.563	0.063	0.063
29	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000

2.4. Three-component mixture design

The three ingredients responsible for the greatest positive impact on several response variables (see Section 3) were used to construct a modified D-optimal mixture design sufficient to satisfy a Scheffé cubic polynomial response surface model (Cornell, 2002). In addition to those needed to satisfy model terms, several points were added to estimate lack of fit (LOF). Several points were duplicated to attain sufficient degrees of freedom (d.f.) to estimate pure error across the design space, provide estimates of block effects, and to attain a near uniform leverage for all points (Weisberg, 1985). The resulting cubic design (Fig. 1) had 2 block, 9 model, 14 lack of fit, and 13 pure error degrees of freedom (Myers and Montgomery, 2002). The LOF diet blends were chosen so that they could be used to satisfy higher order model coefficients if necessary. Treatments were randomly blocked and all blocks were grown in the same environmental chamber.

2.5. Statistical analyses

R^2 is reported as a measure of the amount of variation around the mean explained by the response surface model. However, R^2 can become biased if extraneous model terms are introduced. Therefore, the adjusted-R^2 (R^2_{adj}), which decreases as the number of terms in the model increases if those additional terms do not increase the precision of the model, was calculated as:

$$R^2_{\mathrm{adj}} = 1 - \left(\frac{(n-1)}{(n-p)}\right)(1 - R^2) \quad (1)$$

where n is the sample size and p is the number of model terms. Predicted-R^2 (R^2_{pred}), a measure of the amount of variation in new data explained by the model, was calculated as:

$$R^2_{\mathrm{pred}} = 1 - \left(\frac{\mathrm{PRESS}}{\mathrm{SS_{Total}}}\right) \quad (2)$$

where PRESS is the "prediction error sum of squares" (Allen, 1971). PRESS is calculated by removing a single observation from the response surface model, predicting that response point with the remaining $n-1$ observations, repeating this process for all observations, and then summing the squares of the n PRESS residuals (cf. Myers and Montgomery, 2002).

For each response (e.g., number of adults, average weight) all possible models from the mean to quartic polynomial were

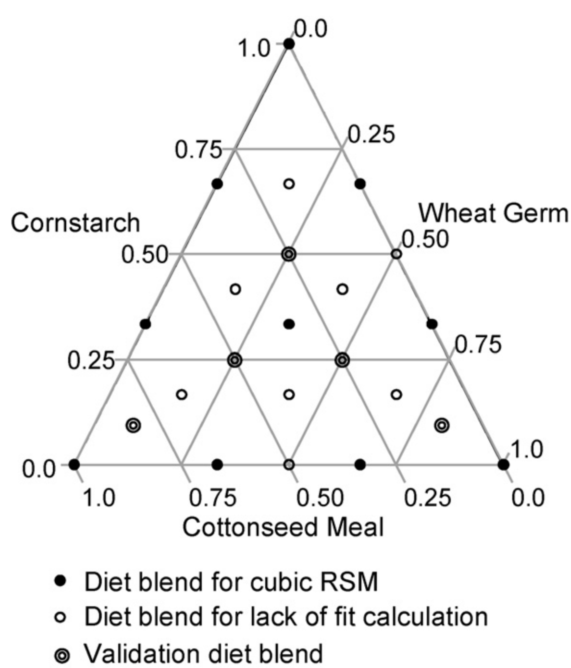

Fig. 1. Design space for a three-component blend experiment showing coordinates of experimental and validation diet blend proportions.

calculated with Design-Expert®. Initial model selection was based on: (a) a lack of any aliased terms; (b) low residuals; (c) a low p-value; (d) nonsignificant lack of fit; (e) a low standard deviation; (f) high R^2, R^2_{adj} and R^2_{pred}; (g) close agreement between R^2_{adj} and R^2_{pred}; and (h) a low PRESS value in relation to the other models. If two or more models were satisfactory then the most parsimonious one was chosen. The selected model was then further evaluated according to a battery of adequacy tests as described by Anderson and Whitcomb (2005, 2007). Normality was determined by examining a normal probability plot of the internally studentized residuals and assuring that the residuals fit closely to a straight line. Constant variance was determined by plotting the internally studentized residuals versus the predicted responses. If the points fell within an interval of ± 3 standard deviations (σ) and exhibited a constant range of residuals across the graph then constant variance was assumed. A Box–Cox plot for selecting the correct power law transformation was created by generating a curve of the natural log of the sum of squares of the residuals (Box and Cox, 1964); a transformation was deemed necessary based on the best lambda value, which is the nadir of the generated curve. "DFFITS" (defined as the change in the predicted value for a point, obtained when that point is left out of the regression) and "DFBETAS" (defined as a normalized measure of the effect of observations on the estimated regression coefficients) plots were used to identify overly influential points (Belsley et al., 1980); points that fell outside $\pm 2\sigma$ were considered suspect (Montgomery et al., 2001; Myers, 1990). Adequate precision of the model was determined by comparing the range of the predicted values at the design points ($\hat{y}$) to the average variance (V-bar) of the prediction (Anderson and Whitcomb, 2005). Potential outlier points were checked with externally studentized "outlier-t" (Weisberg, 1985; Myers, 1990) and Cook's distance (Cook and Weisberg, 1982) graphical plots. The screening design primarily relied upon Cox and/or Piepel trace plots of main effects for analysis (Cox, 1971; Piepel, 1982).

2.6. Three-component model validation

A variety of validation points (Fig. 1) were specified in regions of the design space not included in the initial experiments to empirically assess the usefulness of the predictive capabilities of the proposed RSM model. Larval *D. abbreviatus* were grown on diets representing all of these points and the measured responses were compared to predictions. All responses falling within the 95% prediction interval (PI; Hahn and Meeker, 1991) were considered successful validations.

2.7. Diet optimization

Given the inherent complexity of the diet mixtures, it was necessary to employ a multivariate optimization technique designed specifically to facilitate navigation of n-dimensional response spaces (Nelder and Mead, 1965; Derringer and Suich, 1980). Diet recipes optimized for maximal adult weight, female weight, male weight, numbers of adults and biomass (number of adults multiplied by average adult weight) were calculated with Design-Expert™. This software uses a simplex hill-climbing algorithm in a multi-dimensional pattern search (cf. Press, 1989) that converges at either a stationary point or a design space boundary. The algorithm searches for a combination of factor/component levels that simultaneously satisfies desirability requirements placed on each of the responses and factors/components. These goals are combined into an overall desirability function scaled from 0 to 1. Calculation of diet cost was based on the current (December, 2007) prices of individual diet components and mixes provided by a commercial provider of insect diet ingredients (Bio-Serv, Inc., Frenchtown, NJ).

3. Results

3.1. Screening design

The screening design produced relatively poor linear response surface models with significance of fits (P-values) from 0.11 to 0.34 for the number of adults and average weights, respectively. Given the minimal sampling of the experimental design volume by the screening design approach and the apparent complexity of the responses, the lack of significance for these fits is not surprising. However, trace plots of the Cox-effects, which estimate the effects of increasing the proportion of one component in relation to a reference blend (the standard diet in this case) while the relative proportions of all of the other components are kept constant (Smith, 2005), were consistent and indicated that cottonseed meal and wheat germ had the greatest positive effect on all of the responses measured in this study (Fig. 2). By virtue of the minimal power of the screening design, the quadratic models were all aliased and therefore unreliable; however, fitting the screening design data to this type of model indicated that there might be a positive interaction between cornstarch and cottonseed meal (data not shown). Therefore, cottonseed meal, wheat germ and cornstarch were chosen for further experimentation within a three-component mixture experimental design.

3.2. Three-component mixture design

A summary of the ANOVA, lack-of-fit tests, the best fitting models and the R^2 statistics for several responses are presented in Tables 3 and 4. Some model fits were improved by backward regression and are designated as "reduced". Several responses required transformation as per Box–Cox analyses. The remaining diagnostics were all within acceptable limits, i.e. the data appeared normal and displayed a constant variance, there were no outlier-t points, no points that exceeded a Cook's distance of one, and the predicted versus actual value plots showed close agreement (data not shown). The three R^2 statistics (R^2, R^2_{adj} and R^2_{pred}) were clustered with a difference less than 0.2. Additionally, with the exception of one response, the lack-of-fit tests were not significant (Tables 3 and 4) indicating that additional variation in the residuals could not be removed with better models. The overall models were all highly significant ($P < 0.0001$), indicating significant factor effects on the various responses, and were considered of sufficient quality to navigate the experimental design space and for predicting new observations. The ANOVAs revealed multiple significant terms that are indicative of important component effects and interactions. The regression coefficients are reported in coded terms. Thus, they are directly comparable and provide information on how each term contributes to the shape of the response surfaces.

3.3. Survival and weight of larvae at transfer

The response surface models for the total number and weight of larvae surviving to transfer were significant ($P \leq 0.0002$) but the R^2_{adj} values were 0.45 and 0.62 (data not shown), indicating that the models explained only ca. one-half of the observed variance. Nevertheless, both models indicated that cottonseed meal was the main driver for maximizing both larval survival and weight at the time of transfer (4 weeks). Wheat germ was almost as beneficial as cottonseed meal for maximizing weight at transfer, but was much less important in the total number of larvae surviving to transfer. Cornstarch had a positive but small influence on both of these responses.

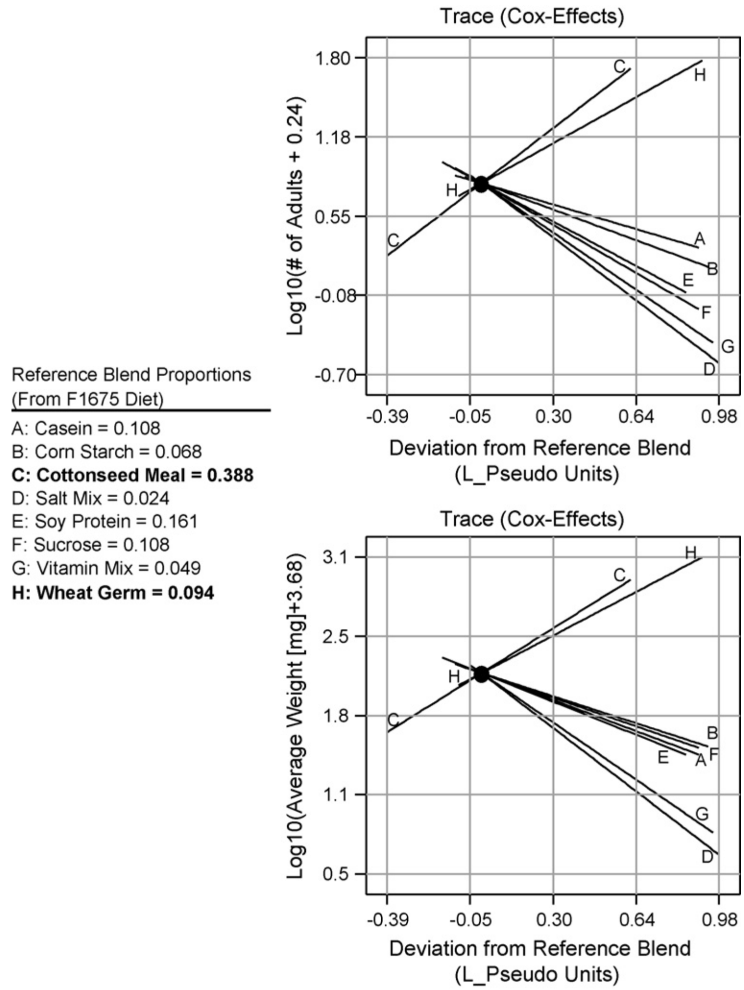

Fig. 2. Cox trace plots showing deviation from the control diet blend of eight-diet components for two-response variables: number of adults (A) and mean weight of adults (B).

Table 3
P-values, regression coefficients and response surface model fitting diagnostic statistics for several *D. abbreviatus* responses to three-component diet blends

	# Pupae *P*-values	Regression coefficients	# Adults *P*-values	Regression coefficients	# Females *P*-values	Regression coefficients	# Males *P*-values	Regression coefficients
Model	<**0.0001**	–	<**0.0001**	–	<**0.0001**	–	<**0.0001**	–
Linear mixture	<**0.0001**	–	<**0.0001**	–	<**0.0001**	–	<**0.0001**	–
CS	–	0.88	–	0.78	–	0.54	–	0.84
CtM	–	4.96	–	4.84	–	3.49	–	3.07
WG	–	4.02	–	3.89	–	3.12	–	2.16
CS × CtM	**0.023**	−4.16	**0.013**	−4.28	**0.012**	−3.90	0.165	−2.44
CS × WG	0.853	−0.31	0.260	−1.75	0.510	−0.92	0.954	0.08
CtM × WG	–	–	–	–	0.631	0.58	0.862	0.22
CS × CtM × WG	–	–	–	–	–	–	–	–
CS^2 × CtM × WG	–	–	–	–	–	–	0.051	−64.41
CS × CtM^2 × WG	–	–	–	–	–	–	0.056	75.57
CS × CtM × WG^2	–	–	–	–	–	–	0.107	−49.72
CS × CtM × (CS − CtM)	–	–	–	–	–	–	–	–
CS × WG × (CS − WG)	<**0.0001**	16.84	<**0.0001**	15.12	<**0.0001**	13.74	–	–
CtM × WG × (CtM − WG)					0.140	4.72	–	–
Lack of fit	0.661		0.565		0.835		0.119	
Model type	Reduced cubic		Reduced cubic		Reduced cubic		Special quartic	
Transformation	Sqrt (#Pup + 0.27)		Sqrt (# Adults + 0.25)		Sqrt (# Females + 0.17)		Sqrt (# Males + 0.13)	
R^2	0.844		0.869		0.857		0.818	
R^2_{adj}	0.810		0.841		0.810		0.745	
R^2_{pred}	0.730		0.774		0.701		0.475	

CS = cornstarch; CtM = cottonseed meal; WG = wheat germ; Sqrt = square root. Significant *P*-values appear in bold.

Table 4
P-values, regression coefficients and response surface model fitting diagnostic statistics for several *D. abbreviatus* responses to three-component diet mixtures

	Mean adult wt. *P*-values	Regression coefficients	*P*-values	Regression coefficients	*P*-values	Regression coefficients	Total biomass *P*-values	Regression coefficients
Model	<**0.0001**	–	<**0.0001**	–	<**0.0001**	–	<**0.0001**	–
Linear mixture	<**0.0001**	–	<**0.0001**	–	<**0.0001**	–	<**0.0001**	–
CS	–	2.10	–	−0.90	–	−2.21	–	11.22
CtM	–	16.52	–	288.05	–	236.65	–	75.50
WG	–	18.54	–	361.37	–	295.18	–	71.29
CS × CtM	<**0.0001**	34.64	<**0.0001**	1027.82	<**0.0001**	619.30	0.364	−35.07
CS × WG	<**0.0001**	33.22	<**0.0001**	654.36	<**0.0001**	631.18	0.587	−17.54
CtM × WG	0.290	2.04	0.171	99.19	0.562	33.62	0.393	25.39
CS × CtM × WG	0.013	−32.60	0.063	−1046.58	0.093	−667.10	0.374	−167.99
CS^2 × CtM × WG	–	–	–	–	–	–	–	–
CS × CtM^2 × WG	–	–	–	–	–	–	–	–
CS × CtM × WG^2	–	–	–	–	–	–	–	–
CS × CtM × (CS − CtM)	<**0.0001**	29.29	**0.005**	851.93	**0.001**	567.25	0.958	3.71
CS × WG × (CS − WG)	<**0.0001**	35.84	<**0.0001**	711.32	0.068	227.31	**0.0004**	247.51
CtM × WG × (CtM − WG)	0.953	0.25	0.787	42.22	0.797	32.74	0.163	93.42
Lack of fit	0.046		0.917		0.534		0.557	
Model type	Cubic		Cubic		Cubic		Cubic	
Transformation	Sqrt (Avg Wt + 3.78)		N/A		N/A		Sqrt (Total biomass + 81.98)	
R^2	0.982		0.954		0.956		0.876	
R^2_{adj}	0.973		0.930		0.933		0.817	
R^2_{pred}	0.941		0.879		0.871		0.666	

Total biomass is calculated as the product of mean female weight and number of adults (# Adults; Table 3). All weights are in mg. CS = cornstarch; CtM = cottonseed meal; WG = wheat germ; Sqrt = square root. Significant *P*-values appear in bold.

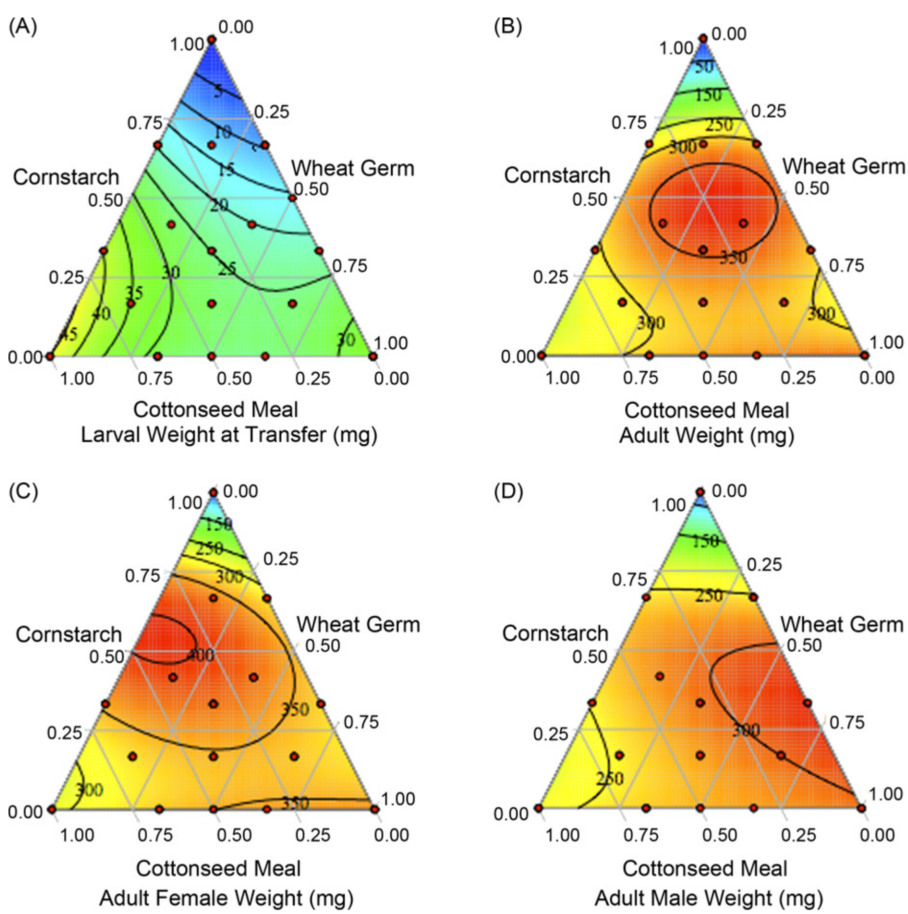

Fig. 3. Predicted three-dimensional surface response plots for four measures of *D. abbreviatus* reared on diets with varying proportions of three major diet components: mean weight of 4-week-old larvae (A), mean adult weight (B); mean adult female weight (C) and mean adult male weight (D).

Table 5
Predicted mean (±95% CI) weight, total biomass, and post-transfer adult survival for *D. abbreviatus* reared on five diets comprised of varying proportions of three major ingredients as defined by response surfaces optimal values (shown in bold) for adult weight (AWt, Fig. 4A), female weight (FWt, Fig. 4B), male weight (MWt, Fig. 4C), biomass (Bm, Fig. 4D) and number of adults (#Ad, Fig. 3B)

Optimal response diet	Component blend[a] (g/100 g)	Mean wt. (mg)			Biomass (g)/100 cups	Adults/100 larvae[b]
		Adults	Females	Males		
AWt	21–10–13	**376 ± 22**	390 ± 24	298 ± 21	5.0 ± 0.7	17 ± 5
FWt	24–17–2	335 ± 31	**406 ± 57**	278 ± 27	3.8 ± 0.9	10 ± 6
MWt	14–0–30	309 ± 32	332 ± 34	**320 ± 28**	2.5 ± 0.8	5 ± 6
Bm	0–33–10	299 ± 30	328 ± 32	260 ± 26	**25.5 ± 2.3**	70 ± 12
#Ad	0–43–0	269 ± 26	288 ± 29	237 ± 24	18.7 ± 1.8	**77 ± 17**
F1675	4–20–5	293 ± 28	306 ± 30	286 ± 18	4.2 ± 0.9	14 ± 10

Similar actual data are provided for the commercial diet F1675 for comparison.
[a] Dry weight of cornstarch, cottonseed meal and wheat germ, respectively.
[b] Reflects survival of post-transfer larvae to adult emergence.

3.4. Survival to pupation, adult emergence and sex ratios

Cottonseed meal and wheat germ were both influential in maximizing the number of larvae that survived to pupation and adulthood (Fig. 4, Table 6). Cornstarch had a minor but positive influence on these responses. There was also a significant antagonistic blending effect between cornstarch and cottonseed meal. Both sexes responded to the diet blends in a similar fashion; the blends that produced higher numbers of adults produced equal and higher numbers of both males and females.

3.5. Weight of adults

Wheat germ was the most important component for maximizing adult weight while cottonseed was a close second (Fig. 3B, Table 5). Cornstarch was only weakly influential by itself, but exhibited several significant blending interactions and exerted positive influence in two and three component blends (Tables 4 and 5). Females were generally heavier than males (Tables 4 and 5) and achieved maximal weight at lesser wheat germ levels and similar proportions of cottonseed meal and cornstarch (Fig. 3C). Males achieved maximal biomass at lesser cottonseed meal levels and greater wheat germ:cornstarch ratios (Fig. 3D).

3.6. Total biomass

Overall, cottonseed meal was the most influential diet component for maximizing total biomass, defined as the total weight of adult insects produced (Fig. 4C, Table 4); wheat germ was also beneficial, while cornstarch had a minor positive effect on the

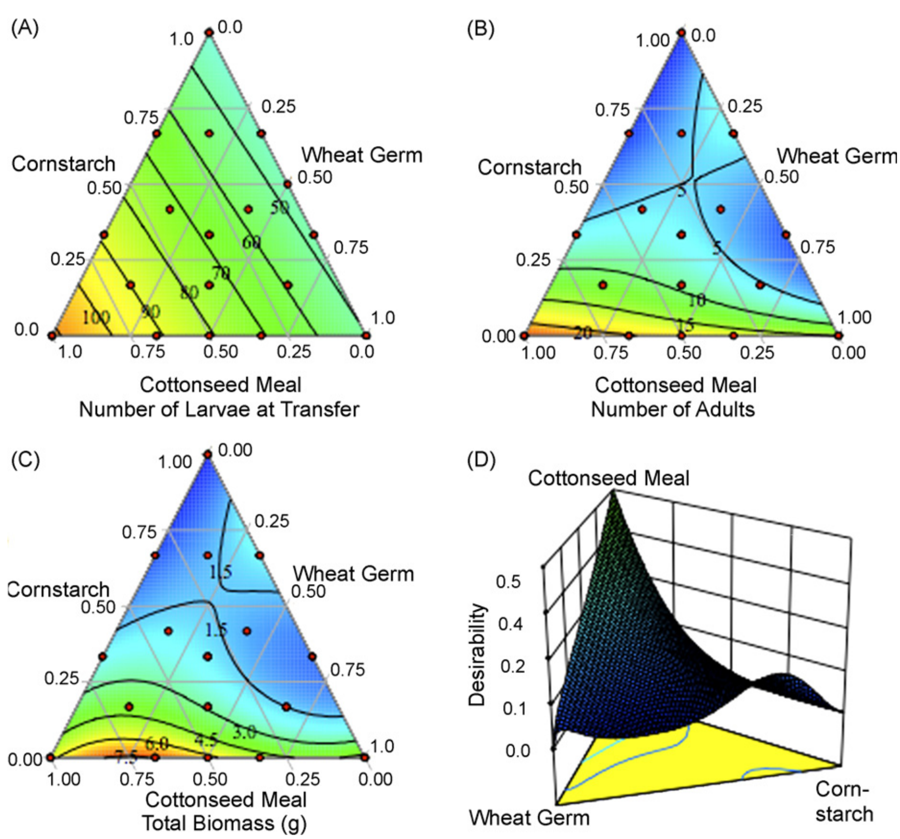

Fig. 4. Predicted three-dimensional surface response plots for three measures of *D. abbreviatus* reared on diets with varying proportions of three major diet components: number of 4-week-old larvae (A), number of adults (B) and total biomass (C). Plot D shows predicted proportions of components optimized to produce maximum number of adults at minimal cost.

Table 6
Predicted mean (±95% CI) larval and pupal survival, cost of diet, and cost to produce a single adult *D. abbreviatus* reared on five diets comprised of varying proportions of three major ingredients as defined by response surfaces optimal values for adult weight (AWt), female weight (FWt), male weight (MWt), biomass (Bm) and number of adults (#Ad)

Optimal response diet	Component blend[a] (g/100 g)	Larval survival[b] (#/cup)	Pupal survival[c] (#/100 larvae)	Pupae (#/kg dry diet)	Cost of dry diet ($/kg)	Total cost (¢/adult)
AWt	21–10–13	4.0 ± 0.7	23 ± 7	105	7.18	6.9
FWt	24–17–2	4.7 ± 0.8	12 ± 8	56	6.84	12.2
MWt	14–0–30	3.2 ± 1.0	8 ± 8	37	7.85	21.5
Bm	0–33–10	6.5 ± 1.0	75 ± 13	337	8.12	2.4
#Ad	0–43–0	7.5 ± 1.4	81 ± 19	370	7.91	2.2
F1675	4–20–5	4.3 ± 0.8	23 ± 7	103	8.32	8.5

Similar actual data are provided for the commercial diet F1675 for comparison.
[a] Dry weight of cornstarch, cottonseed meal and wheat germ, respectively.
[b] Number of larvae surviving per diet cup from neonate to transfer at 4 weeks.
[c] Number of larvae pupating per 100 post-transfer larvae by 4 months after transfer.

greatest total biomass responses (Table 4). The effect of greater survivability of weevils reared on diets containing a greater proportion of cottonseed meal was influential in skewing the total biomass response surface away from the region of maximal weight (Fig. 4C). None of the blending/interaction terms for this response was significant.

3.7. Model validation and diet optimization

All measured responses using the diets formulated for model validation fell within the 95% prediction intervals of the various models developed during this study, which indicates that these models are capable of reliably interpolating responses. Optimized diet recipes for a variety of responses are detailed in Tables 5 and 6. An example of a diet optimized for maximum survival to adult and minimum cost is presented in Fig. 4D.

4. Discussion

Entomologists who rely on artificially-reared insects often wish for a better diet but assume they lack the time or resources to pursue the matter. Diet optimization research is considered by many researchers to be tedious and convoluted and is generally under-appreciated (Cohen, 2004). The experimental complexity of identifying optimal diet blends can vary from relatively simple, as in the case of the early screwworm (*Cochliomyia hominivorax*) diet (water, beef, blood and formalin) (Melvin and Bushland, 1940) to complex mixtures such as the Debolt (1982) diet for *Lygus hesperus* (19 components) or the "simplified" replacement for the Debolt diet developed by Cohen (2000) (16 components). We suspect many ingredients are included in insect diets because other successful diets have done so and/or the primary motive is to, 'just get something that works.' In these cases, any diet that supports growth, even if suboptimal, allows the researcher to pursue what are perceived to be more important research objectives. This pragmatic acceptance of colony-reared insects for research applications may, in some cases, cloud the validity of such research.

For many insect species, including *D. abbreviatus*, diets are adapted or derived from blends that have been shown to produce satisfactory results for related species. Beavers (1982) reported the recipe for what became the F1675 diet (15 ingredients including vitamin and salt mixes, excluding water and agar). In that article, Beavers alluded to diets developed for the boll weevil, the plum curculio and the cerambycid *Dectes texanus*, but no explanation was provided as to how the particular combination and proportion of ingredients acceptable to *D. abbreviatus* were determined. Since that publication, *D. abbreviatus* has been produced for ca. 25 years by the USDA and more recently by the state of Florida with no modification of the original diet. Many thousands of colony-reared *D. abbreviatus* have been used in experiments spanning fields such as biological control, genomics, plant resistance, ecology, chemical ecology, and others. During that period, no one, ourselves included, has examined the artificial diet and questioned basic assumptions about the quality of colony-reared insects or the applicability of results obtained with artificially-reared *D. abbreviatus* to insects from the field. Cohen (2004) suggested the low status of insect diet development work and a lack of appreciation for rearing specialists as factors that contribute to lower quality research and hinder technological advances in the field.

Cohen (2004) emphasizes varying one factor at a time (OFAT) in his book on insect diets (cf., p. 120) as the only suitable method for determining the effect of a single diet ingredient, thereby ignoring the importance of interactions. As R. A. Fisher (1971) pointed out, the OFAT approach is not very helpful in research where no prior knowledge is available about whether the factors to be studied exert their effects independently or are related, through interactions, to variations in other factors. "If the investigator, in these circumstances, confines his attention to any single factor, we may infer either that he is the unfortunate victim of a doctrinaire theory as to how experimentation should proceed, or that the time, material or equipment at his disposal is too limited to allow him to give attention to more than one narrow aspect of his problem" (Fisher, 1971, p. 94). To further quote Fisher from the same source (p. 102), "... any conclusion ... has a wider inductive basis when inferred from an experiment in which the quantities of other ingredients have been varied, than it would have from any amount of experimentation, in which these had been kept strictly constant." Fisher and others eloquently make the case for multivariate designs. Fortunately, recent evolution of computer hardware and software simplifies application of efficient experimental designs that vary multiple ingredients simultaneously.

We set ourselves the goal of answering a set of questions related to the rearing of *D. abbreviatus*, a major polyphagous pest of the Caribbean and the southern U.S.: could the diet be simplified and made more economical; could diets be identified that would satisfy different individual criteria or combinations of criteria; could the diet be designed to produce adult weights similar to field-collected insects; and could we describe mathematically the multidimensional space encompassing all combinations of principal diet ingredients, thereby relating the organism to its diet components in Euclidian space? If this last goal is possible, we may be able to define an insect's response to continuously varying diet ingredients, allowing the researcher to adjust the proportions of diet ingredients to produce insects of a determined quality for a particular purpose.

Our results illustrate the power of using mixture experiment principles and response surface methodology to analyze diet formulations. The benefits of this approach include avoidance of confounding effects of parallel changes in ingredients and the ability to study interaction effects of diet ingredients over the entire application space (Ruohonen et al., 2003). It is evident that diet F1675 is unnecessarily complex and includes ingredients (casein, soy protein isolate and sucrose) of little or no value at least as measured by the response variables employed in our screening design. The Cox-effect traces (Fig. 2) resulting from the screening design showed that varying the proportions of these ingredients had little effect on diet performance for multiple responses compared with the reference blend. Thus, the primary result of the screening experiment is a simplified diet with greater productivity and reduced cost (see Tables 1 and 6). The three-component study further demonstrated that cornstarch and wheat germ are not necessarily desirable components if greater survival of lower weight individuals is desired (Table 5). The diet recipe optimized for maximum survival at least cost includes cottonseed meal as the major nutritive component, eliminates cornstarch and wheat germ (Fig. 4D and Table 6) and greatly reduces levels of casein, soy protein and sucrose.

Our results demonstrated that researchers interested in rearing *D. abbreviatus* can produce adult insects within a range of values for a desired character or combination of characters by selecting the corresponding component blend (e.g., high survival and low cost, Fig. 4D). It is therefore possible for researchers to use specific physiological objectives (e.g., maximizing female weight, often a correlate of fecundity) to optimize the diet recipe.

The decision by Beavers (1982) to use a high proportion of cottonseed meal in his diet for *D. abbreviatus* appears to have been fortuitous. He also included several ingredients (casein, cornstarch, soy, sucrose) that do not enhance F1675 diet performance. The approach of Beavers (and others) to insect diet development was likely based on intuition and subject matter knowledge. This resulted in a diet capable of supporting growth and maturation of *D. abbreviatus*, but is by no means optimal for any of the responses measured in the experiments described here. The ability to test the large number of permutations resulting from a diet blend of 14 components (plus agar and water) was beyond the analytical capabilities of all but the most mathematically astute researchers at that time. The approach presented here relies on advanced computing power coupled with user-friendly software and provides a logical framework for analyzing complex mixtures of diet components.

The diet blend optimized for adult weight (Fig. 3B) is predicted to produce adult *D. abbreviatus* that weigh 28% more compared with adults reared on the commercial F1675 diet (Table 5). It is notable that the blends that optimize individual adult weights (AWt, FWt and MWt in Table 5) also result in low survival compared with those blends (Bm and #Ad) predicted to yield adults of more modest proportions. We have observed that colony-reared *D. abbreviatus* tend to be larger (Hall and Alessandro, 2005) and are not as active as field-collected individuals in laboratory and greenhouse behavioral assays. This raises the possibility that larval nutrition conferred by the commercial F1675 diet may result in larger insects with an impaired behavioral repertoire. Karino et al. (2004) demonstrated that the body size of Japanese horned beetles, *Allomyrina dichotoma*, is determined by larval nutrition and that larger males employ different tactics to attain reproductive success compared with smaller males. In their study, the larval nutritional environment determined adult behavior. Our ongoing hypothesis is that colony-reared and field-collected *D. abbreviatus* adults of comparable size will evince similar behavior in laboratory assays.

We have identified a set of response-optimized diets for *D. abbreviatus* that contain fewer ingredients than the current commercial diet, but we did not attempt to de-convolute the vitamin and salt mixes. From the screening design, it was evident that small quantities of these mixes are required but inclusion of larger quantities, not surprisingly, was deleterious. It would be possible to apply the same experimental approach used here to study the effect of individual vitamin or salt components.

Our objective was to optimize the existing F1675 diet by studying the contributions of individual diet ingredients. Because we manipulated diet components to create a design space and not protein, carbohydrate and lipid levels directly, we were interested to know, post hoc, how well our design points had sampled the experimental region of an unconstrained mixture design defined by the three macronutrient axes (cf. Ruohonen and Kettunen, 2004). Because we varied ingredients, the design space (unshaded area in Fig. 5) was constrained within the experimental space by the amounts of protein, carbohydrate and lipid contained in the diet components. Because none of the diet ingredients had substantial lipid content (0.3, 5.2 and 11.5% lipid for cornstarch, cottonseed meal and wheat germ, respectively), the lipid dimension was poorly sampled (Fig. 5). If greater lipid content were desired, a source other than the ingredients tested here would have to be used. However, diet with greater lipid content might fall outside of the application space given a priori knowledge about the feeding habits of larvae. When we overlaid our response variables on the macronutrient simplex (Fig. 5), the fits were poor (results not presented) and gave no additional insight. Within the highly constrained lipid dimension, there was very little response as measured by survival and weight gain. Rather, the greatest responses were orthogonal to protein concentration; protein content and particularly proteins found in cottonseed meal appeared to be driving survival and weight. Our data suggested that the effect of protein was source-specific since our response variables were significantly less affected by soy protein isolate and casein (Fig. 2). It appears that characteristics of cottonseed meal other than protein:carbohydrate ratio were responsible for its positive effect on development of *D. abbreviatus*. This component-specific effect is further reinforced by observing the near overlap of the F1675 diet and the predicted diet

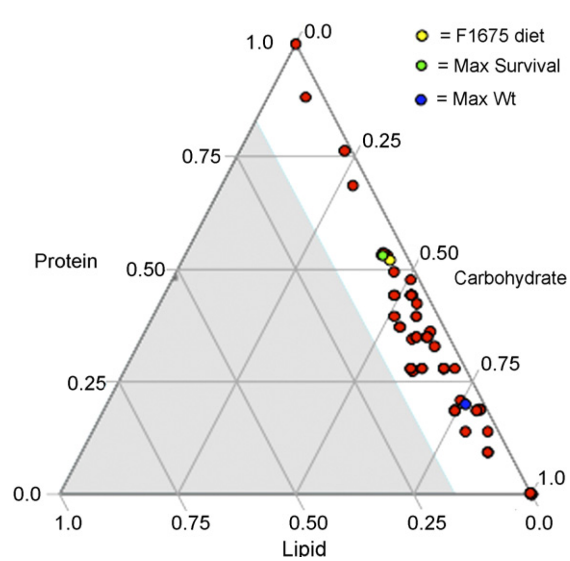

Fig. 5. The unshaded area indicates area sampled by design points within the experimental region of an unconstrained mixture design defined by macronutrients. The design space was constrained within the experimental space by the amounts of protein, carbohydrate and lipid contained in the diet components. The design points are largely orthogonal to the protein:carbohydrate ratio with the high lipid region largely unsampled. The yellow circle indicates the commercial BioServ F1675 diet; the green and blue circles refer to the predicted optimal diets based on survival (s) and adult weight (w), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

for maximal survival within the macronutrient design space (Fig. 5). Greater survival on the optimized diet compared with the commercial blend was not an effect of macronutrient proportions.

Lee et al. (2008) suggested that in *Drosophila*, an increasing proportion of protein may reduce adult longevity while an intermediate protein:carbohydrate ratio results in maximal lifetime egg production. It is important to note that we did not measure fitness as a response variable, as defined by lifetime egg production and adult lifespan (cf. Lee et al., 2008). Our measures were confined to larval survival, and larval and adult weight. Harari et al. (1999) reported a positive relationship between male *D. abbreviatus* size and reproductive success due to the ability of large males to disrupt matings by smaller males, their ability to intromit more quickly than small males, and positive size-assortative mating resulting in large males preferentially mating with large, and presumably more fecund females. However, our objective was not to produce the most fit individuals, but rather to define a diet to optimize production of adult weevils of a normative weight compared with field-collected insects for experimentation related to mating behavior and host localization. In our case, reproduction is not limiting, but the costly and prolonged developmental period of larval *D. abbreviatus* constrains our ability to produce healthy adults for experimentation.

Sirot et al. (2006) suggested that behavioral traits influence postcopulatory reproductive success in *D. abbreviatus*. We have observed that *D. abbreviatus* reared on diet F1675 are heavier and larger, and that they tend to be less active in laboratory assays compared with field-collected adults (SL, unpublished data). Within the design space defined by the three principal drivers of cornstarch, cottonseed meal and wheat germ, the high cottonseed meal blend (#Ad, Table 5) predicted high survival of weevils of lower mass than those produced on blends containing cornstarch and wheat germ. Nonetheless, larvae reared on the high cottonseed meal blend emerged as larger adults compared with *D. abbreviatus* collected in citrus groves in our region (St. Lucie County, FL). We were unable to identify a diet blend within our design space that produced adults of comparable weight to field-collected *D. abbreviatus* while maintaining a high rate of survival (adult emergence). Our ongoing objective is to produce a diet that is nutritionally sound but produces large numbers of weevils of comparable weight to field-collected insects. In hindsight, we might have included cellulose, a non-nutritive filler, as a variable in our designs based on our previous experience with the F1675 diet (Lapointe et al., 2003). In our current research related to the chemical ecology of *D. abbreviatus*, our goal is to produce insects that respond in laboratory and greenhouse behavioral assays in a manner consistent with field-collected adults. By varying the diet blend based on our response surface models, we intend to compare the effect of diet and adult size on activity in behavioral bioassays and thereby test our hypothesis that colony-reared *D. abbreviatus* of comparable size to field-collected also behave in bioassays in a manner comparable to field insects.

Modest changes to insect diets can result in major economic benefits. In the case of the screwworm, Chaudhury and Skoda (2007) recently reported that replacing one ingredient with a less expensive one would reduce production costs of the Mexico-U.S. Commission for the Eradication of Screwworm by $100,000 annually. In their work, two cellulose fiber-based diets were compared to a standard diet in one experiment, and the effect of varying the total amount of diet was compared in a separate OFAT design. With only a small increase in experimental runs, it would be possible to fully evaluate all six components of the screwworm diet, identify main drivers, and quantify main effects and interactions in a statistically robust manner. Because a multi-variate geometric approach would completely characterize the six-component design space, currently unexplained regions of the design space with further economic savings could be located. Or, if no such region were found, it would be known that the true optima for the six components had been achieved.

The experimental design implemented in this study still required considerable effort to execute, especially for an insect with a long developmental period such as *D. abbreviatus*. But compared with an OFAT approach, the multivariate/geometric approach allowed for simultaneous testing of multiple components without eliminating important interaction effects in a highly efficient manner while also providing important information on interaction/blending effects. We believe that many insect-rearing programs would benefit from the application of these methods to situations where diet optimization is desired. This approach is applicable more broadly to any insect diet problem that can be conceptualized as a mixture problem.

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